# Bone Sialoprotein Supports Breast Cancer Cell Adhesion Proliferation and Migration Through Differential Usage of the αvβ3 and αvβ5 Integrins

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Bone sialoprotein (BSP), a secreted glycoprotein found in bone matrix, has been implicated in the formation of mammary microcalcifications and osteotropic metastasis of human breast cancer (HBC). BSP possesses an integrin-binding RGD (Arg-Gly-Asp) domain, which may promote interactions between HBC cells and bone extracellular matrix. Purified BSP, recombinant human BSP fragments and BSP-derived RGD peptides are shown to elicit migratory, adhesive, and proliferative responses in the MDA-MB-231 HBC cell line. Recombinant BSP fragment analysis localized a significant component of these activities to the RGD domain of the protein, and synthetic RGD peptides with BSP flanking sequences (BSP-RGD) also conferred these responses. The fibronectin-derived RGD counterpart, GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro), could not support these cellular responses, emphasizing specificity of the BSP configuration. Although most of the proliferative and adhesive responses could be attributed to RGD interactions, these interactions were only partly responsible for the migrational responses. Experiments with integrin-blocking antibodies demonstrated that BSP-RGD-induced migration utilizes the αvβ3 vitronectin receptor, whereas adhesion and proliferation responses were αvβ5-mediated. Using fluorescence activated cell sorting, we selected two separate subpopulations of MDA-MB-231 cells enriched for αvβ3 or αvβ5 respectively. Although some expression of the alternate αv integrin was still retained, the αvβ5-enriched MDA-MB-231 cells showed enhanced proliferative and adhesive responses, whereas the αvβ3-enriched subpopulation was suppressed for proliferation and adhesion, but showed enhanced migratory responses to BSP-RGD. In addition, similar analysis of two other HBC cell lines showed less marked, but similar RGD-dependent trends in adhesion and proliferation to the BSP fragments. Collectively, these data demonstrate BSP effects on proliferative, migratory, and adhesive functions in HBC cells and that the RGD-mediated component differentially employs αvβ3 and αvβ5 integrin receptors. J. Cell. Physiol. 176:482–494, 1998. 

Although bone is the most common site of human breast cancer (HBC) metastasis, relatively little is known about the specific molecular mechanisms responsible for colonization and preferential growth of HBC cells in bone (Yoneda et al., 1994). Breast cancer metastases are predominantly osteolytic, and the tumor cells progressively destroy bone either directly through tumor-derived proteases or activation of bone-residing enzymes (Ellon and Mundy, 1978), or indirectly, by secreting factors such as parathyroid hormone-related protein (PTHrP), which recruits and stimulates osteoclasts to resorb bone (Mundy, 1991; Bouizar et al., 1993; Clohisy et al., 1996). Osteolysis also can be influenced by a variety of local growth factors, hormones and cytokines, and tumor cells may,

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for example, be able to stimulate local production and activity of prostaglandins and/or vitamin D, leading to increased bone resorption (Orr et al., 1993). In order to metastasize, breast cancer cells must migrate out of the vasculature and into the bone marrow cavity and proliferate in the bone environment, and it has been suggested that HBC cell invasion and growth in bone also may be facilitated by attachment to and interaction with bone extracellular matrix components, such as collagen I, fibronectin and laminin (Yoneda et al., 1994).

Bone sialoprotein (BSP) is an acidic, sulfated glycoprotein that is primarily secreted by osteoblasts and osteoclasts and is thought to take part in bone formation by binding to hydroxyapatite (Fisher et al., 1983; Bianco et al., 1991). Recent work also has indicated that BSP stimulates proliferation of mouse preosteoblast cells (Zhou et al., 1995). In normal bone, BSP is one of the most abundant noncollagenous proteins and is usually associated with newly synthesized osteoid, lending support to a role in bone mineralization (Hunter et al., 1993). The complete nucleic acid sequences of human, rat, mouse, and cow, as well as a partial cDNA sequence for pig BSP have been determined and encode an ~ 80-kD, highly modified protein with a conserved integrin-binding RGD (Arg-Gly-Asp) tripeptide and three polyglutamic acid domains thought to confer hydroxyapatite-binding abilities. Rat and human BSP are the most highly conserved, having a 70% sequence homology, including the RGD domain, which is necessary for the cell-binding abilities of BSP as well as several other extracellular matrix proteins, including vitronectin and fibronectin (Oldberg et al., 1988a; Fisher et al., 1990; Shapiro et al., 1993; Chenu et al., 1994; Young et al., 1994). Additionally, the amino acids directly flanking the RGD are almost identical in rat and human BSP. From previous reports, it is likely that the RGD domain of BSP mediates the attachment of osteosarcoma cells and osteoclasts in vitro (Oldberg et al., 1988b; Helfrich et al., 1992; Ross et al., 1993). The RGD domain of osteopontin (OPN), a closely related bone matrix protein, has been implicated in osteoclast-mediated bone resorption, initiation of cellular signaling pathways, and increased adhesion and migration of HBC cells (Bautista et al., 1994; Chellaiah et al., 1996; Xuan et al., 1995). Aside from its RGD-mediated activities, OPN also appears to have non-RGD domains that promote cell attachment (Van Dijk et al., 1993), and a similar, non-RGD-related cell adhesion mechanism has been evidenced for BSP (Mintz et al., 1993; Stubbs et al., 1995). Recently, bone sialoprotein, which is implicated in osteoclast attachment and bone resorption (Raynal et al., 1996), has been localized to human breast cancer in association with abnormal microcalcifications (Bellahcene et al., 1995), and significantly higher BSP expression levels were found in primary breast lesions from patients who eventually developed bone metastases (Bellahcene et al., 1996a). Similarly, OPN is expressed in a variety of primary carcinomas; however, in situ hybridization studies showed tumor-associated macrophages to be the source of OPN in these lesions (Brown et al., 1994). Further studies demonstrated that the MDA-MB-231 human breast cancer cell line could recognize synthetic peptides encompassing the specific RGD sequence of human BSP (Van der Pluijm et al., 1996), and in an in vitro adhesion assay, these peptides were able to inhibit tumor cell adhesion to bone matrix, suggesting that BSP may support adhesion of human breast cancer cells to the bone through RGD-binding integrins.

The integrins, a large family of $\alpha/\beta$ heterodimeric transmembrane proteins, mediate cell-cell and cell-substratum adhesion and interact with many extracellular matrix components, including fibronectin, vitronectin, collagens, and laminin (Sheppard, 1996). Integrins are often expressed in high numbers on the cell surface and, depending on cell type, can sometimes bind multiple ligands, or show alternative specificity to influence different cellular responses to the same ligand (Sheppard, 1996). Recent work also has demonstrated a role for integrins in initiation of signaling pathways in response to extracellular matrix (Clark and Brugge, 1995). At this time, there is an abundance of research detailing changes in expression or function of integrins that occur upon malignant transformation and which may help to explain their altered phenotype and uncontrolled growth rates. For example, in mammary adenocarcinomas, there is evidence for decreased expression of the $\alpha2\beta1$ and $\alpha5\beta1$ integrins (Zutter et al., 1993, 1995), and recently, a $\beta1$ integrin blocking antibody was shown to dramatically normalize a malignant mammary phenotype (Weaver et al., 1997). Similarly, the $\alpha v\beta3$ integrin has been implicated in the increased adhesion, motility, and metastasis of large cell lymphoma and in the growth of human melanoma cells (Montgomery et al., 1994; Yun et al., 1996). Current data relate integrin expression to tumor cell attachment and migration as well as proliferation. In one such study, Agrez and colleagues (1994) demonstrated enhanced in vitro and in vivo proliferative capacity of a human colon carcinoma cell line expressing $\alpha v\beta6$.

BSP contains an RGD domain that is thought to support integrin-mediated attachment of certain cells in normal bone. Adhesion molecules, including the $\alpha v\beta3$ integrin and the integrin-like osteoclast functional antigen (OFA), have been implicated in both attachment of osteoclasts to bone matrix and resorption of bone (Davies et al., 1989). Human prostate carcinoma cells were demonstrated to adhere to bone matrix via the $\alpha2\beta1$ integrin (Kostenuik et al., 1996), whereas breast carcinoma cells adhere to bone via the $\alpha v\beta3$ integrin (Liapis et al., 1996).

Because malignant as well as normal cells use cell surface integrins to interact with the bone matrix, we hypothesized that BSP may influence HBC cell metastasis to bone by imparting upon the tumor cells an increased capacity to adhere to, proliferate in, and migrate through the bone matrix. The present study demonstrates that BSP can indeed invoke positive growth, attachment, and migratory responses by breast carcinoma-derived cells and that whereas the proliferative and adhesive responses appear to be $\alpha v\beta5$-mediated, the $\alpha v\beta3$ integrin seems to mediate the migratory response. This is the first time that BSP has been reported to influence tumor cell proliferation and migration, broadening the scope of its involvement in breast cancer and bone metastasis. To the best of our knowledge, this is the first description of an interaction between the $\alpha v\beta5$ integrin and BSP. These data imply that different cellular responses to the RGD domain

of BSP may be regulated by separate populations of integrins and that an interaction between breast cancer cells and the bone matrix is important for bone metastasis.

## MATERIALS AND METHODS

### Cell culture

The MDA-MB-231 and MDA-MB-435 human breast cancer cell lines were originally derived from pleural effusions and obtained from ATCC (Rockville, MD), and the LCC15-MB cells were recently isolated from the femoral metastasis of a breast cancer patient (Thompson et al., 1997). All cell lines were grown in Richter's Improved Minimum Essential Medium (IMEM, Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) at 37°C with 5% $CO_2$. Routine screening for mycoplasma was conducted by the Lombardi Cancer Center Tissue Culture Core Facility.

### Reagents

**BSP, recombinant fragments and cyclic RGD peptides.** Rat bone sialoprotein (BSP) was isolated from the UMR-106-01-BSP rat osteosarcoma cell line as previously described (Mintz et al., 1994). The recombinant BSP fragments synthesized in *Escherichia coli* (amino acids 258-317; designated BRB 9 and 10) (Stubbs, 1996), and the cyclic and linear BSP-derived RGD peptides, CBA4 and BA3 (EPRGDNYR; denoted CNB and CP3 in Van der Pluijm et al., 1996), respectively, were synthesized and kindly provided by Dr. Frank Robey (NIDR, NIH, Bethesda, MD). Figure 1 illustrates the different BSP preparations that we used in this study, highlighting the RGD consensus sequence as a reference point in each form. Note that the recombinant fragments BRB 9 and 10 are identical with the exception of the RGD substitution to KAE in BRB 10.

**Antibodies.** The integrin blocking antibodies against αv (MAb L230; 10 μg/ml), αvβ3 (LM609, MAb 1976; 10 μg/ml), and αvβ5 (MAb clone P1F6, mouse ascites; 1:2,000 dilution) were obtained from ATCC, Chemicon International (Temecula, CA) and Gibco BRL, respectively. Antibody concentrations were those that gave maximal blocking responses in preceding titration experiments. The vitronectin used in the adhesion assays was a generous gift from Dr. Steven Akiyama (NIDR, NIH, Bethesda, MD), and the Alamar Blue vital dye was from Biosource International (Camarillo, CA).

### Cell migration assay

Migration experiments were performed in triplicate essentially as previously described (Thompson et al., 1992), but adapted for the 48-well Boyden chamber apparatus (Neuroprobe, Cabin John, MD). EHS tumor-derived type IV collagen in 0.5 M sodium acetate (50 μg/filter), a gift from Dr. Hynda Kleinman (NIDR, NIH), was applied to polycarbonate filters (12.0 μm pores, PVP-free, Costar, Cambridge, MA), and allowed to dry. Full-length rat BSP, recombinant BSP fragments, or RGD peptides (1 μM) were reconstituted in phosphate-buffered saline (PBS, Gibco BRL), diluted in IMEM supplemented with 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO), and used as the chemoattractant in the bottom chamber. IMEM plus 0.1% BSA

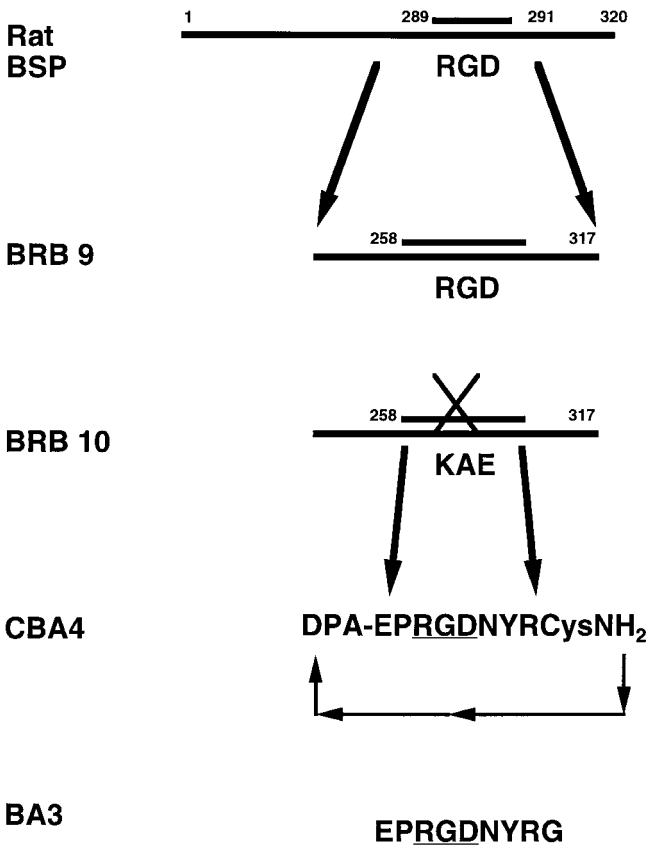



Fig. 1. Schematic depiction of full-length rat bone sialoprotein (BSP) and the different human BSP-derived constructs used in this study. BRB9 and BRB10 are recombinant fragments of human BSP with and without (KAE substituted for RGD) the RGD domain, respectively, and CBA4 and BA3 are the cyclic and linear forms of the human BSP-derived RGD peptide.

(IMEM-BSA) and IMEM with 10% FBS (IMEM-FBS) were used as baseline and positive controls, respectively. Cells were harvested with trypsin (Gibco BRL), washed twice with serum-free IMEM, resuspended in IMEM-BSA, and added to the top chamber at a density of 15,000 cells/well. Chambers were incubated in a humidified incubator at 37°C in 5% $CO_2$ for 18 h. The cells that had traversed the filter and spread on its lower surface were stained with Diff-Quik (American Scientific Products, McGaw Park, IL) and quantitated by counting the number of cells per nine representative fields at a magnification of 20×. Data presented are an average (with standard deviation) number of cells per nine representative microscopic fields.

### Cell proliferation assay

Proliferative responses (anchorage-dependent) were assessed in triplicate in 96-well plates with full-length BSP, recombinant BSP fragments, and RGD peptides diluted in IMEM-BSA to the concentrations indicated above. Similar to the migration assay, IMEM-BSA and IMEM-FBS were used as baseline and positive controls, respectively. Cells were harvested with trypsin and plated in IMEM-FBS at a density of 1500 cells/well at 37°C, 5% $CO_2$. On the following day, the media

was removed, the cells washed twice with serum-free IMEM, and the experimental media (proliferative agent in IMEM-BSA) added. Cell number was assayed after 1, 3, and 5 days using Alamar Blue (Biosource International), a nontoxic dye that living cells can reduce from a blue to red color. After incubation with the dye for 24 hours, extent of proliferation as measured by absorbance at 570 and 600 nm was determined using a 96-well plate ELISA reader (Dynatech MR700, Dynatech Laboratories, Chantilly, VA).

### Flow cytometric analysis

Cells (50,000) were harvested with trypsin in the log phase of growth, washed with PBS, and resuspended in 100 μl 3% BSA/IMEM with the anti-integrin antibodies αvβ3 (0.2 μg) and αvβ5 (1:500 dilution), followed by incubation for 1 hr on ice. After washing twice with 1 ml IMEM/3% BSA, 100 μl FITC-conjugated goat anti-mouse IgG in IMEM/3% BSA was added to the cells and incubated for 1 hr on ice. Finally, cells were again washed twice with IMEM/3% BSA and analyzed for surface integrin expression by flow cytometry (FACS-plus, Becton Dickinson, San Diego, CA). For cell sorts, single cells enriched for either αvβ3 or αvβ5 (top 5%) were selected, cloned into 96-well plates, and grown for 2–3 weeks through a single passage, after which they were subjected to a second negative single cell sort to exclude remaining cells that retained expression of the other integrin. Although populations were relatively enriched for one particular integrin, there was continued low expression of the other integrin. Sorted cells were used within 2 weeks of the second sort, and selective integrin expression profile was relatively stable for 1 month.

### Attachment assay

Attachment assays were performed essentially as previously described (Thompson et al., 1993) in triplicate in 96-well plates precoated with either vitronectin (1 μg/ml), BSP fragments, or BSP-derived RGD peptides (1 μM) in PBS for 1 hr at 37°C. The coating solution was then replaced with 50 μl of 3% BSA/PBS added to each well for 30 min, 37°C, to block non-specific binding sites. The wells were then washed three times with PBS. Meanwhile, the cells were harvested with trypsin and $2.5 \times 10^5$ cells/ml were washed and resuspended in IMEM/BSA. The cell suspension was incubated with or without integrin blocking antibodies for 1 hr at 37°C before being added (total volume = 100 μl) to each precoated well and incubated for 1 hr to allow for attachment. The supernatant containing the unattached cells was then removed and the attached cells rinsed gently with PBS and stained with 50 μl of 0.05% crystal violet in 25% methanol for 5 min. The plate was rinsed three times by immersion in water and allowed to dry at room temperature. The incorporated dye was dissolved in 100 μl 0.1 M sodium citrate in 50% ethanol and measured by reading absorbance at 540 nm with a 96-well plate ELISA reader.

## RESULTS

### MDA-MB-231 responses to BSP and BSP fragments

We first monitored MDA-MB-231 cell migration, proliferation, and attachment responses to rat BSP as well as to the recombinant human BSP fragments. As shown in Figure 2A, MDA-MB-231 cells migrated readily across the filter toward BSP and both BRB 9 and 10. BRB 10 (with a mutated RGD domain) evokes only a slightly smaller migratory response than BRB 9, suggesting that BSP confers both RGD and non-RGD-mediated stimulation of cell migration. As seen later (see Fig. 6), the RGD-mediated component is augmented in cells enriched for αvβ3 integrin expression. Possible nonspecific effects due to the *E. coli* vehicle elements were unlikely since a recombinant BSP-derived fragment spanning an upstream segment of the protein was unable to elicit migration (not shown).

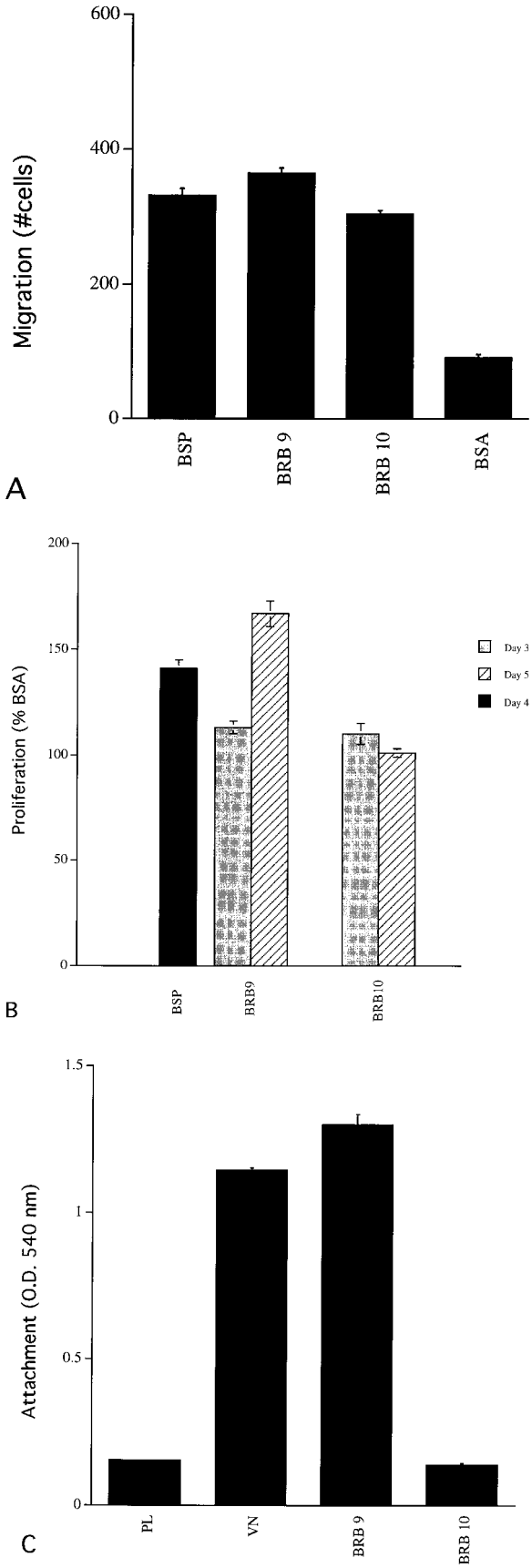
MDA-MB-231 proliferative responses to rat BSP were also positive, showing ~40% stimulation over basal proliferation in 0.1% bovine serum albumin (Fig. 2B). However, the BSP RGD consensus appears to be more important for MDA-MB-231 proliferation than migration, since BRB 10 was much less effective than BRB 9 in increasing proliferation. These effects were seen after 5 days, but were not evident at day 3. Purified BSP was only tested at day 4 due to its limited availability.

Using BSA-blocked plastic and vitronectin as negative and positive controls, respectively, we further examined RGD involvement in attachment of MDA-MB-231 cells to BSP (Fig. 2C). The cellular adhesion response profile mirrored proliferation, but with more pronounced HBC cell attachment to BRB 9 than BRB 10. Cell attachment toward vitronectin is shown as a point of reference. These results suggest that the RGD domain of BSP is required for proliferation and adhesion to BSP, whereas both RGD and non-RGD motifs are involved in MDA-MB-231 cell migration toward BSP.

### MDA-MB-231 responses to RGD peptides

To characterize further the RGD-mediated responses, we examined MDA-MB-231 responses to BSP-derived RGD peptides. The cyclic (CBA4) and linear peptides (BA3) contain the RGD consensus sequence flanked on either side by 2 and 3 BSP amino acids, respectively, and have recently been shown to inhibit MDA-MB-231 cell adhesion to bone sections (Van der Pluijm et al., 1996). We first employed these peptides in an attempt to block BSP responses to show RGD specificity, but, instead, discovered that the peptides themselves induced the cells to migrate and proliferate. Figure 3A demonstrates that these peptides selectively stimulate cell migration, the cyclic CBA4 peptide being almost twice as potent as the linear BA3 form. In contrast, no effect was seen with the linear fibronectin-derived GRGDSP peptide.

MDA-MB-231 proliferation (Fig. 3B) and attachment (Fig. 3C) experiments also showed direct BSP-RGD responses, and again, the cyclic BSP-derived RGD peptide was more active than the linear form in both cases. As with migration, neither the fibronectin-derived GRGDSP peptide nor the GRGDSP control peptide supported cellular proliferation or adhesion, indicating the BSP-RGD specificity of these responses. The increased effectiveness of the cyclic form of the BSP-RGD peptide (CBA4) as compared to its linear form (BA3) implies that the tertiary conformation of the BSP molecule is important in conferring migratory, proliferative, and




adhesive responses, as was indicated previously (Van der Pluijm et al., 1996).

### Effects of integrin-blocking antibodies on cellular responses

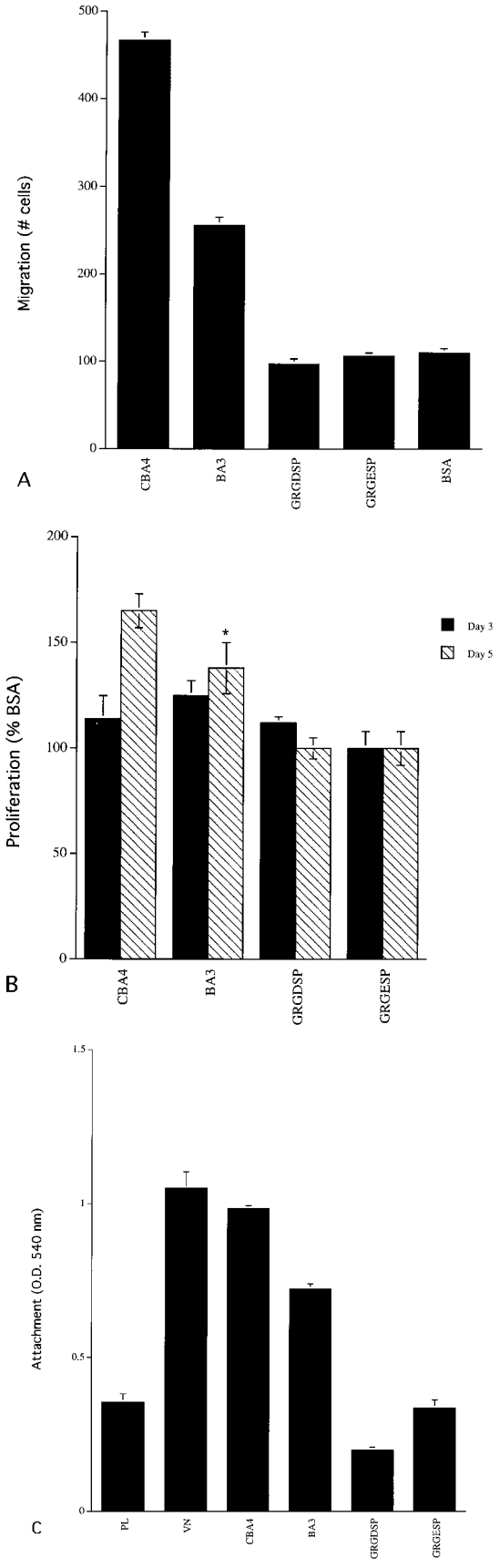
The observation of novel RGD-mediated BSP responses in MDA-MB-231 cells raised the question of which cell surface integrins might be responsible for these effects. Because both osteopontin and BSP have been shown to interact with αv-containing integrins, we first tested an anti-αv antibody. As seen in Figure 4A–C, the L230 antibody decreased migration, proliferation, and adhesion of the HBC cells to CBA4 by approximately one-half and to BA3 by a lesser extent. Further delineation using blocking antibodies to either αvβ3 or αvβ5 integrins demonstrated that migration responses were not inhibited by the αvβ5 blocker, whereas proliferative and adhesive responses were significantly decreased ($P > 0.05$). When the RGD migration response was examined in isolation with CBA4, we saw that it was largely αvβ3-mediated (Fig. 4A). As seen previously, BRB10 elicited non-RGD-mediated cellular migration (Fig. 4B), whereas proliferation and adhesion were predominantly RGD-mediated (Fig. 4B,C). Shown in Figure 4C is the ability of the αv and αvβ5 antibodies to block attachment of the MDA-MB-231 cells to BRB 9, indicating that the peptides are comparable to the recombinant BSP fragments. Additionally, we were able to block the attachment to vitronectin with both αv and αvβ3 blocking antibodies (not shown). We confirmed the presence of these integrins on MDA-MB-231 cells by flow cytometric analysis and found the αvβ3 integrin to be expressed at lower levels (Fig. 5A). It is therefore apparent that the MDA-MB-231 cells can utilize different integrins for different cellular responses to BSP-RGD sequences (αvβ5-mediated cell proliferation and attachment and αvβ3-mediated migration).

### MDA-MB-231 selectants

To confirm and extend our study of the integrin-specific responses in MDA-MB-231 cells, we used a fluorescence-activated cell sort (FACS) to select two subpopulations of MDA-MB-231 cells, one enriched for αvβ3 integrin expression and the other for αvβ5 integrin expression (designated 231αvβ3 and 231αvβ5, respectively). The integrin profiles of these two sublines are shown in Figure 5B,C. The integrin enrichment of these subpopulations were stable in culture for up to 4 weeks as indicated by FACS analysis.

We repeated our previous experiments on the integrin-selected subpopulations and found that the 231αvβ3 cells had an increased migratory capacity when compared to their 231αvβ5 counterparts (Fig. 6A), consistent with the previous blocking studies. The 231αvβ5 selectants showed no evidence of RGD-dependent migration, whereas the 231αvβ3 selectants showed increased RGD-dependent migration compared to pa-

Fig. 2. MDA-MB-231 cell migration (**A**), proliferation (**B**), attachment (**C**) to rat BSP and recombinant human BSP fragments BRB9 and BRB10. BSA (bovine serum albumin), PL (plastic), and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells in a representative experiment.

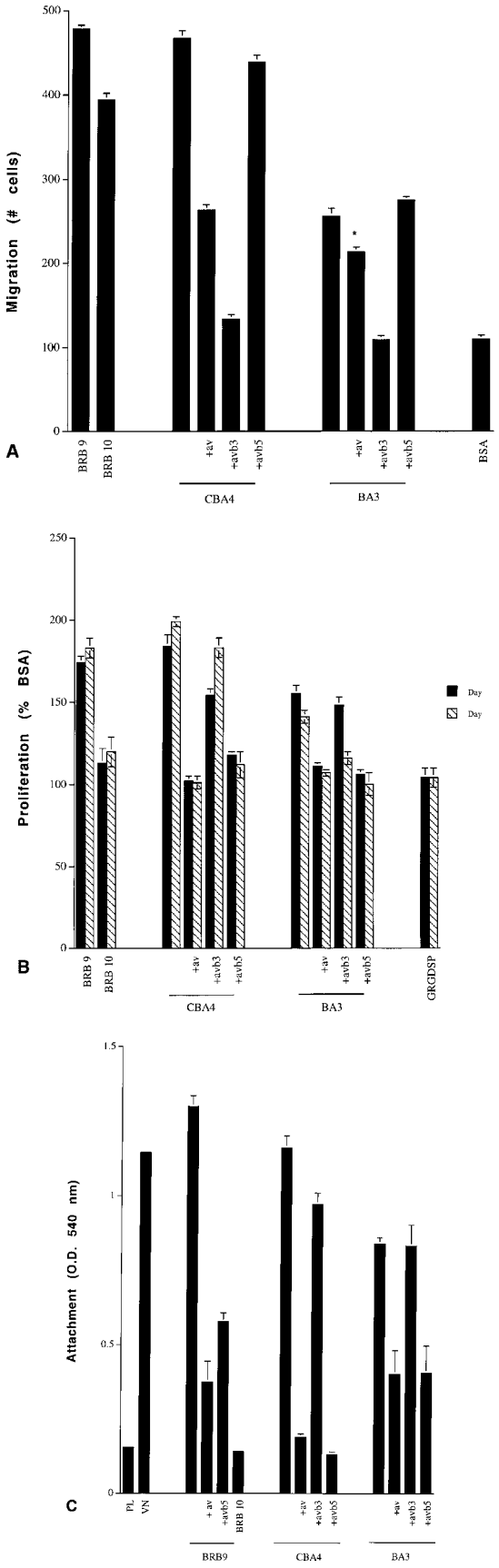



rental cells (BRB9 > BRB10), and this could be blocked most effectively with the αvβ3 antibody. The 231αvβ5 cells showed a more pronounced proliferative response to CBA4 (compared to parental cells) on day 5, and this could be effectively attenuated by αv or αvβ5, but not by αvβ3 antibodies. In contrast, the 231αvβ3 subpopulation showed a very low initial proliferative response that was not blocked by αvβ3 (Fig. 6B). This was consistent with the RGD-dependent proliferation of the 231αvβ5 selectants in response to BRB9 but not BRB10. Although not shown, day 3 proliferation responses were similar. Results from the adhesion experiments with the MDA-MB-231 subpopulations were similar to those from proliferation experiments, but more pronounced. Whereas both 231αvβ3 and 231αvβ5 populations show increased cell attachment to the BSP-derived RGD peptides, attachment could be inhibited by the αvβ5 blocking antibody only in the 231αvβ5 cells (Fig. 6C). Blocking antibodies to αvβ3 were unable to inhibit attachment of αvβ3 cells, suggesting an alternative mechanism for BSP-mediated attachment and proliferation in the αvβ3 selectants. As previously shown, adhesion appeared to be RGD-dependent since attachment of both selectant populations to BRB10 was significantly diminished ($P < 0.05$) as compared to BRB9. As with parental cells, GRGDSP was unable to induce significant migration, proliferation, or attachment in these sublines, showing the BSP specificity of this RGD response. Overall, the results with these selected subpopulations confirm the differential αvβ3 and αvβ5 integrin usage for migration, proliferation, and adhesive responses of MDA-MB-231 cells to BSP.

### BSP RGD-induced responses in other HBC cell lines

In order to investigate the cell specificity of our results, we examined similar response profiles of two additional breast cancer cell lines, MDA-MB-435 and LCC15-MB. When analyzed for integrin expression by flow cytometry, the LCC15-MB and MDA-MB-435 cell lines both showed expression of the αvβ3 and αvβ5 integrins (Fig. 5D,E); however, the analysis also revealed higher αvβ3 levels in these two cell lines as compared to the MDA-MB-231 cell line, altering the ratio between the two integrins. In contrast to the MDA-MB-231 cells, both of these cell lines showed very little inherent migration, even to serum-supplemented medium, a potent positive stimulant of migration, such that migration responses of these cells toward BSP could not be evaluated. This has been demonstrated previously for the MDA-MB-435 cells (Thompson et al., 1992), and also seen to some extent in the LCC 15-MB cells (Sung et al., 1998). Most likely, this poor response in LCC15-MB is due to the poor adhesion of these cells for collagen type IV (Sung et al., 1998). However, as illustrated in Figure 7A,B, the MDA-MB-435 and LCC15-MB cell

Fig. 3. MDA-MB-231 cell migration (**A**), proliferation (**B**), attachment (**C**) to BSP-derived RGD peptides CBA4 and BA3 and fibronectin-derived RGD peptide (GRGDSP) and corresponding mutant form (GRGESP). BSA (bovine serum albumin), PL (plastic), and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells in a representative experiment, and proliferation to BA3 is significantly lower than that obtained by CBA4 (day 5). *$P <$ 0.05 in two-sided t-test.




lines did show positive proliferative and adhesive responses toward the BSP fragment BRB 9. These responses appeared to be RGD-dependent since the non-RGD containing BRB10 was ineffective in increasing proliferation or adhesion of the two cell lines.

Figure 7A shows that BSP-induced proliferation of the LCC15-MB and MDA-MB-435 cell lines, at approximately equivalent levels compared to the MDA-MB-231 cells, is at least partially RGD-dependent, since responses were seen to BRB 9 but not to BRB 10. Cellular proliferation of the MDA-MB-435 cells was also stimulated in response to the BSP-derived RGD peptides and blocked by the αvβ5 blocking antibody, and again, lack of proliferation toward a fibronectin-derived RGD peptide (GRGDSP) further signified the importance of a BSP specific RGD peptide in stimulating proliferation of the MDA-MB-435 cells. In contrast, the LCC15-MB cells have a much diminished proliferative response to the BSP-RGD peptides, which was not effectively blocked by the integrin antibodies, suggesting an additional mechanism of BSP-induced growth in these cells.

We also analyzed adhesion of the two additional cell lines and showed that the MDA-MB-435 and LCC15-MB cells, similar to the MDA-MB-231 cells, attached in an RGD-dependent manner to BRB 9, but not to BRB 10, which lacks the RGD tripeptide (Fig. 7B). Although the BSP-derived RGD peptides were effective in stimulating adhesion of these cells, levels of adhesion were somewhat reduced, especially in the LCC15-MB cell line. In addition, whereas MDA-MB-435 adhesion to BSP peptides was blocked by the αvβ5 blocking antibody, this was not the case for LCC15-MB cells (Fig. 7B). Overall, these data demonstrate that although BSP does appear to confer general RGD-mediated proliferative and adhesive properties on a number of different breast cancer cells, the extent and mechanism of stimulation may vary from one cell type to another, depending on the presence and ratio of αvβ3 and αvβ5, and perhaps other cell surface integrins.

## DISCUSSION

Although the mechanisms and regulation of carcinoma metastasis are not well understood, significant progress has been made in recent years. Most notably, cellular attributes such as adhesion, degradation, migration, and proliferation have been formulated into the metastatic cascade, through which the primary tumor cells must pass to establish at a site of secondary growth (Bernstein and Liotta, 1994). The central findings in this work are that BSP can support cellular migration, proliferation and attachment of the human breast cancer cells and that a significant part of these responses is mediated by interactions between the BSP-RGD motif and the αvβ5 (proliferation, adhesion)

Fig. 4. Effects of αv, αvβ3 and αvβ5 integrin blocking antibodies on MDA-MB-231 cell migration (**A**), proliferation (**B**), attachment (**C**) to BSP-derived RGD peptides CBA4 and BA3. Recombinant BSP fragments BRB9 and BRB10 are also shown as reference points. BSA (bovine serum albumin), GRGDSP (fibronectin-derived RGD peptide), PL (plastic), and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells of a representative experiment, and αv-blocked migration to BA3 is significantly lower than migration toward BA3 alone. $^*P < 0.05$ in two-sided t-test.

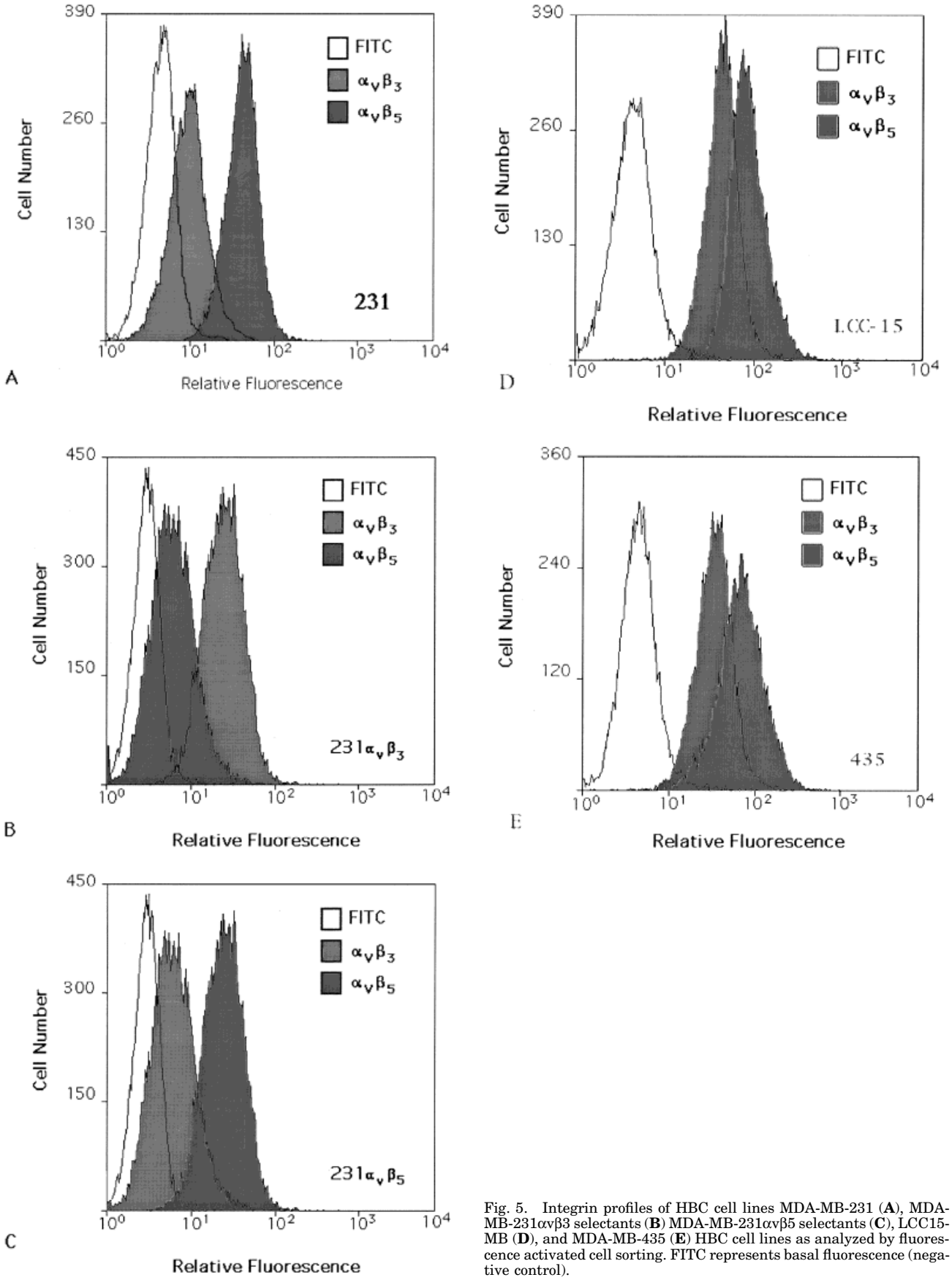



Fig. 5. Integrin profiles of HBC cell lines MDA-MB-231 (**A**), MDA-MB-231αvβ3 selectants (**B**) MDA-MB-231αvβ5 selectants (**C**), LCC15-MB (**D**), and MDA-MB-435 (**E**) HBC cell lines as analyzed by fluorescence activated cell sorting. FITC represents basal fluorescence (negative control).

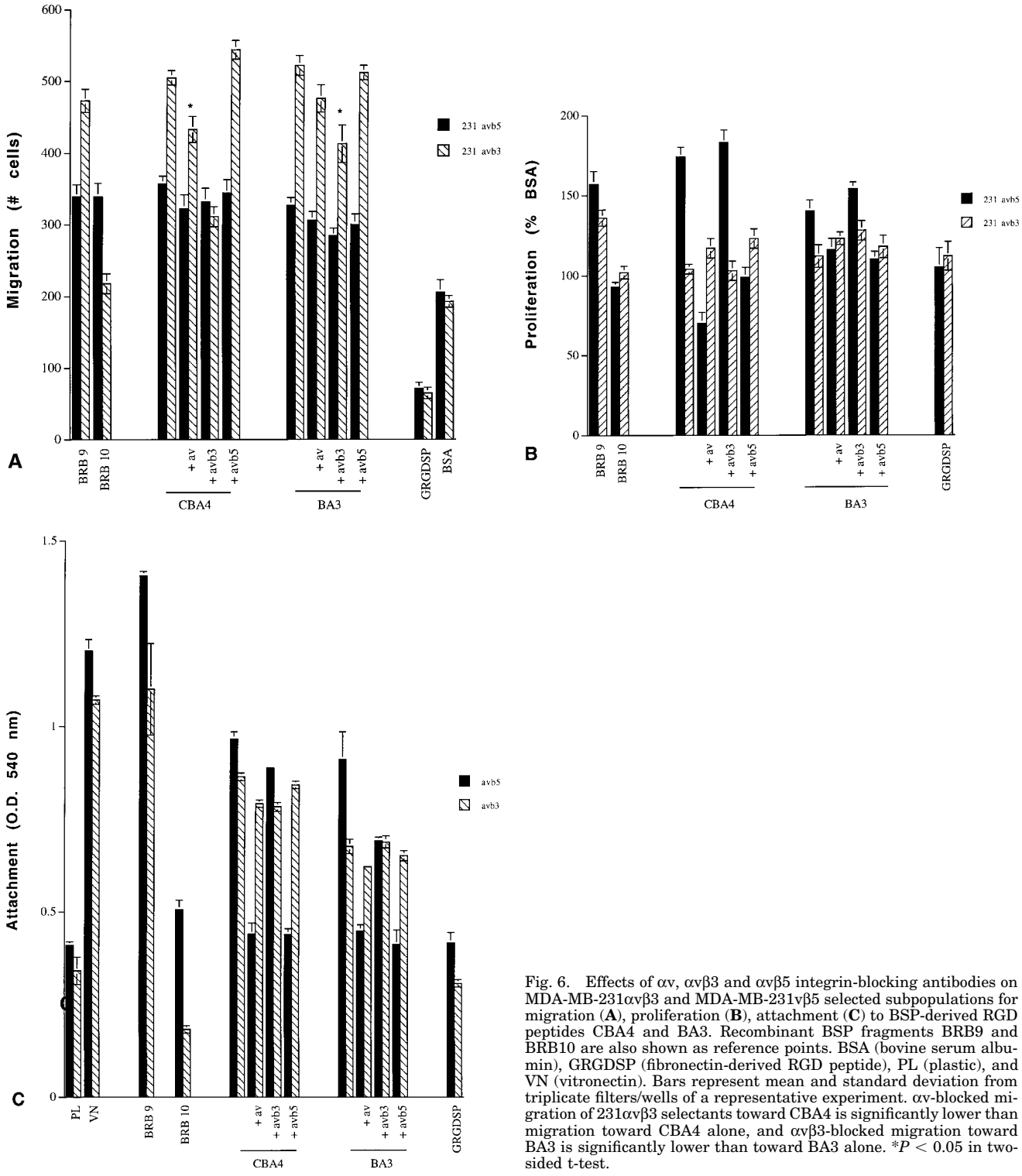



Fig. 6. Effects of αv, αvβ3 and αvβ5 integrin-blocking antibodies on MDA-MB-231αvβ3 and MDA-MB-231vβ5 selected subpopulations for migration (**A**), proliferation (**B**), attachment (**C**) to BSP-derived RGD peptides CBA4 and BA3. Recombinant BSP fragments BRB9 and BRB10 are also shown as reference points. BSA (bovine serum albumin), GRGDSP (fibronectin-derived RGD peptide), PL (plastic), and VN (vitronectin). Bars represent mean and standard deviation from triplicate filters/wells of a representative experiment. αv-blocked migration of 231αvβ3 selectants toward CBA4 is significantly lower than migration toward CBA4 alone, and αvβ3-blocked migration toward BA3 is significantly lower than toward BA3 alone. $^*P < 0.05$ in two-sided t-test.

or αvβ3 (migration) integrins, respectively. In the case of migration, an additional and perhaps more potent non-RGD stimulus exists in BSP and the combined effects of these remain to be tested. Indeed, in further studies (Sung et al., unpub.), BSP transfection of the MDA-MB-231 cells did upregulate baseline migration. The responses seen here were initially established with purified rat BSP followed by more detailed studies using recombinant human BSP fragments expressed in *E. coli* and BSP-specific RGD-containing peptides. We found that the cyclic form of the BSP-RGD peptide conferred migratory, proliferative, and adhesive properties

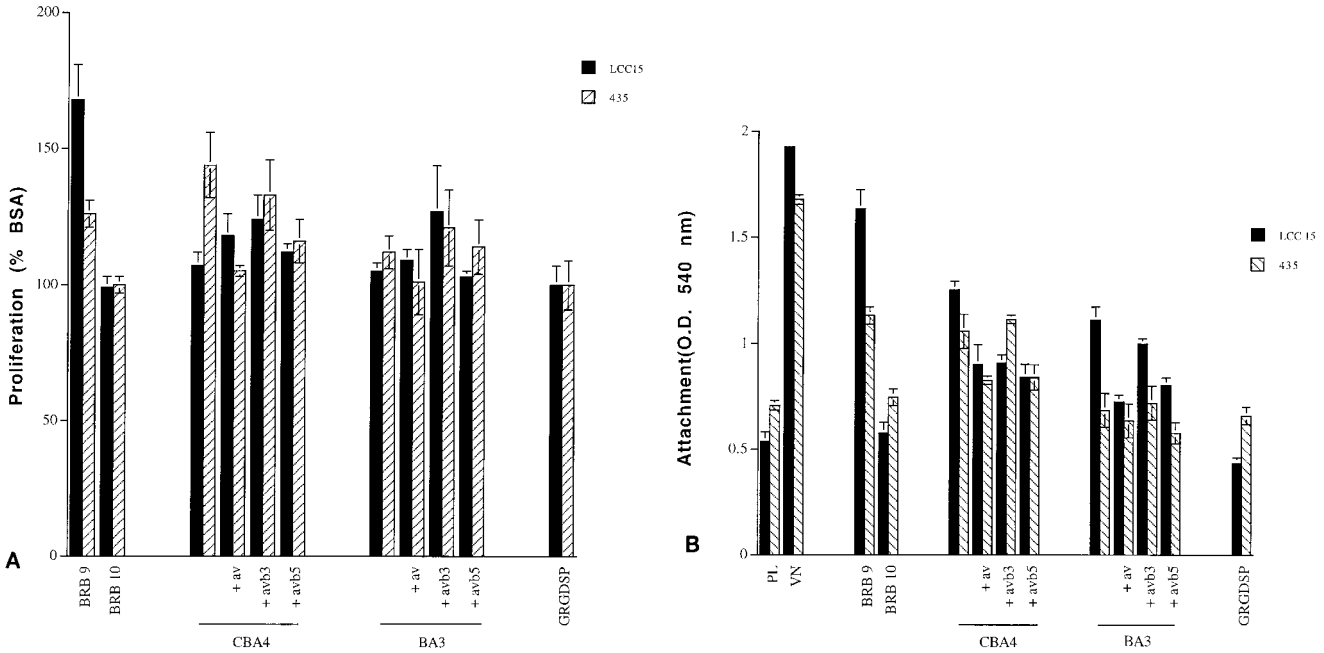



Fig. 7. Effects of recombinant BSP fragments BRB9 and 10 and the BSP-derived RGD peptides CBA4 and BA3 on MDA-MB-435 and LCC15-MB HBC cell proliferation (**A**) and adhesion (**B**). Also shown are effects of αv, αvβ3 and αvβ5 integrin-blocking antibodies on the responses elicited by the BSP peptides. GRGDSP is a fibronectin-derived RGD peptide, PL (plastic) and VN (vitronectin).

comparable to the RGD component of full length rat BSP, and although additional responses mediated by other BSP domains are likely, we have indicated a significant role of the integrin-binding RGD domain of BSP in these responses. Blocking studies using anti-integrin antibodies and subpopulations enriched for integrin expression showed αvβ5 to be the primary integrin involved in adhesion and proliferation responses, and αvβ3 to be more important for the RGD-mediated cellular migration responses in MDA-MB-231 cells.

Similar studies using two additional HBC cell lines (MDA-MB-435 and LCC15-MB) show that BSP-RGD-induced proliferation and adhesion appear to be a general phenomenon among these cell lines and, at least in the case of MDA-MB-435, could be shown to involve the same differential integrin usage. However, proliferative and adhesive responses of the LCC15-MB cell line to BSP peptide were markedly diminished, and we were unable to implicate the αvβ5 integrin in blocking studies. Such differences are not completely surprising, given the diverse origins of the HBC cells as well as variety of integrins expressed on one cell type. As shown in the results, the LCC15-MB and MDA-MB-435 cell lines express higher levels of αvβ3 integrin than the MDA-MB-231 cells, and, indeed, the MDA-MB-435 cell line revealed proliferative and adhesive responses that may more closely resemble the αvβ3-selected MDA-MB-231 cells. The expression profiles of other integrins may also differ among the three cell types, and could presumably result in stimulation of different signal transduction pathways (Ruoslahti et al., 1994) and may explain our inability to inhibit LCC15-MB proliferation and adhesion with the αvβ5 antibody. Nevertheless, our results using different breast cancer cell lines provide evidence for a range of BSP-induced cell responses in more than one cell line. Positive BSP immunoreactivity is present in almost 80% of primary breast lesions, and whereas emerging literature correlating BSP expression in primary breast tumors with disease outcome suggests an autocrine mechanism of primary tumor invasion, BSP abundance in bone also supports a paracrine mode of action in facilitating bone metastasis (Bellahcene et al., 1995, 1996b). Our results demonstrating the effects of BSP fragments on three different HBC cell lines help to define a possible role for BSP in tumor invasion and metastasis.

The adhesion, migration, and proliferation of the MDA-MB-231 HBC cell line in response to BSP could be important at both the primary and metastatic (bone) sites of tumor growth. Two previous studies have associated the interaction of BSP and αv-containing integrins with bone metastasis activities. Adhesion of the MDA-MB-231 cell to rat vertebral bone slices could be blocked by the same BSP-derived RGD peptide as we used here, suggesting that metastasizing breast cancer cells may use BSP in order to attach to the bone matrix and also that the BSP-specific sequences flanking the RGD domain are important in the adhesive functions of BSP (Van der Pluijm et al., 1996). Another study demonstrated that transfection of 293 cells with either αvβ3 or αvβ5 promoted cellular attachment to BSP (Duong et al., 1996). Our studies have revealed a novel proliferative response of HBC cells to BSP, as well as cellular attachment and migratory responses. We have also demonstrated an important interaction between the BSP-RGD domain and specific integrins in MDA-MB-231 cell proliferation, migration, and adhesion, behaviors that may contribute to the effect of BSP on metastasis. Matrix degradation, another important

event in cancer progression, was not examined here, but Teti and colleagues (1996) demonstrated a role for BSP in activation of matrix metalloproteinase (MMP-2) and the urokinase plasminogen system. It is important to note, however, that non-RGD responses to the BSP fragments also were seen in all three HBC cell lines examined and could figure prominently in breast cancer cell metastasis.

The integrin family has long been implicated in cell-cell adhesion, but only recently was shown to provide cells with migration, growth, and differentiation signals. Integrin expression and binding specificity varies greatly from cell to cell and, in addition, also may be regulated within one cell type by molecules such as growth factors and cytokines that affect cell proliferation and differentiation (Ruoslahti et al., 1994). As such, integrins participate in a variety of normal and abnormal physiological processes including wound healing, angiogenesis, and tumor invasion (Pignatelli et al., 1992). Recently, numerous immunohistochemical and in situ hybridization studies have demonstrated irregular integrin expression in breast tumor progression and development of metastasis. For example, loss of $\alpha 2\beta 1$ and $\alpha 5\beta 1$ integrins resulted in increased tumorigenicity of poorly differentiated mammary carcinoma (Pignatelli et al., 1992; Zutter et al., 1993, 1995), whereas $\alpha v\beta 3$ integrin expression has been associated with invasiveness of mammary carcinomas and the MDA-MB-231 HBC cell line (Tondravi et al., 1996; Van Muijen et al., 1996). In analysis of breast cancer integrin expression in 22 bone biopsies containing breast cancer metastasis, Liapis and colleagues (1996) discovered that whereas $\alpha v\beta 3$ expression was mixed in the primary tumors, it was abundant in all breast cancer cells that had metastasized to bone, alluding to a bone-specific induction of the $\alpha v\beta 3$ integrin. Several $\alpha v\beta 3$-binding matrix proteins including BSP, OPN, vitronectin and collagen I have been isolated from skeletal tissue and may actually participate in selective establishment of tumor cells expressing $\alpha v\beta 3$ (Ingram et al., 1993; Grzesik and Robey, 1994). Our results suggest that $\alpha v\beta 5$ and $\alpha v\beta 3$ are two integrins that may promote breast cancer cell interactions with bone matrix proteins and, more specifically, that use of the $\alpha v\beta 5$ integrin contributes to BSP-induced proliferation and adhesion, whereas the $\alpha v\beta 3$ integrin is at least partly responsible for migration responses.

The differential use of integrins for adhesion, migration, and proliferation is not unusual, having been studied previously in several different cell lines. A study using neural crest cells that express three different $\alpha v$ integrins demonstrated that $\alpha v\beta 1$ primarily mediates adhesion to vitronectin, whereas migration of these cells was mediated by both $\alpha v\beta 3$ and $\alpha v\beta 5$. The cellular localization of these integrins corresponded to their functions, with $\alpha v\beta 1$ located mostly at focal adhesion sites, whereas the other integrins were diffuse on the cell surface (Delannet et al., 1994). Similarly, human pancreatic carcinoma cells, which normally only express $\alpha v\beta 5$ and adhere to, but do not spread or migrate on vitronectin, did so upon subsequent transfection of the $\beta 3$ integrin subunit (Leavesley et al., 1992). Although much of the integrin research centers around cellular adhesion, spreading, and migration, there are also recent studies in which integrin ligation has been shown to stimulate cell growth. Expression of both $\beta 1$ and $\beta 5$ integrins in CHO cells promotes spreading of CHO on fibronectin, whereas expression of $\beta 1$ alone resulted in increased proliferative response to fibronectin (Pasqualini and Hemler, 1994). Likewise, introduction of the $\alpha v\beta 6$ integrin into colon carcinoma cells increased their proliferative capacity both in vivo and in vitro, and, interestingly, this required a specific 11 amino acid domain of the $\beta 6$ subunit that is not necessary for other functions of the $\beta 6$ cytoplasmic domain such as adhesion and focal contact localization (Agrez et al., 1994). These studies support a general scheme in which a number of different integrins, which traditionally function in cell adhesion, also are involved in migration and proliferation, depending on the cellular localization and cell type in which they are expressed. Finally, two additional studies that focus on the BSP-related protein OPN also indicate differential integrin usage. A recent kinetic and biochemical study of OPN receptors has determined that both $\alpha v\beta 1$ and $\alpha v\beta 5$ can act as receptors for this bone matrix protein and can bind OPN with a similar affinity as the more well known $\alpha v\beta 3$ integrin (Hu et al., 1995); however, different functional uses for each integrin were not examined. Furthermore, Liaw and colleagues (1995) recently showed that although all three $\alpha v$ integrins can mediate smooth muscle cell adhesion to OPN, migration was dependent on only the $\alpha v\beta 3$ integrin. These studies further support our current findings, which show that $\alpha v\beta 3$ is responsible for breast cancer cell migration, whereas adhesion and proliferation to BSP are mediated via the $\alpha v\beta 5$ integrin. Future experiments could be directed toward dissection of the intracellular events that may follow integrin ligation to result in either growth, adhesion, or migration of the cells.

The intracellular second messenger cascades that follow cell surface integrin clustering and/or activation are now becoming better understood. For example, both integrin aggregation and ligation were required to induce intracellular accumulations of focal adhesion kinase (FAK) accompanied by tyrosine phosphorylation, and integrin aggregation alone could induce prompt transmembrane accumulation of a number of signal transduction molecules (Miyamoto et al., 1995a,b). However, Chen and others (1994) demonstrated that fibroblast attachment to a synthetic fibronectin-derived RGD peptide is sufficient for mitogen-activated protein kinase (MAPK) activation and subsequent translocation from the cytosol to the nucleus. In osteoclasts and also in human melanoma cells, adhesion to OPN via $\alpha v\beta 3$ led to rapid production of phosphoinositides, tyrosine kinase activity, and activation of the c-src oncogene, respectively (Hruska et al., 1995; Chellaiah et al., 1996). Whereas only full-length OPN was tested in the study with melanoma cells, a small RGD-containing OPN peptide could elicit myoinositol incorporation in osteoclasts. Furthermore, this effect appeared to depend on RGD, since RGE peptides could not stimulate the synthesis of phosphoinositides. Finally, and extremely relevant to our study, is the finding that a BSP-derived RGD peptide can induce an increase in intracellular free calcium ions in osteoclasts and that this event may occur through the vitronectin receptor (Shankar et al., 1995). Since we have demonstrated breast cancer cell responses to BSP peptides in vitro,

it may be possible that similar intracellular calcium fluctuations or mobilization of other signaling molecules are involved in integrin-stimulated growth and migration. Interestingly, the increase in free calcium in the osteoclasts could be either coupled or uncoupled to a concomitant cellular retractile event by structurally modifying the peptide. Indeed, other mechanisms including "inside-out signaling" in which internal cellular events lead to modification of integrin function may also exist, and as seen with our experiments with the LCC15-MB and MDA-MB-435 cell lines, different integrins on a single cell type have been shown to activate separate intracellular pathways (Leavesley et al., 1993).

In summary, we have demonstrated that the bone matrix protein, BSP, can stimulate the migration, proliferation, and adhesion of breast cancer cells through its RGD domain and that migration occurs through usage of the αvβ3 integrin, whereas αvβ5 appeared to mediate proliferation and attachment. Given the abundance of BSP in primary breast tumors as well as in bone, these breast cancer cell responses could be instrumental in both increased escape of cells from the primary tumor and in their successful establishment as bone metastasis. In addition, since BSP has been shown to stimulate bone resorption in vitro (Raynal et al., 1996), integrin-mediated attachment to bone matrix also may provide a direct mechanism for tumor cell osteolysis of bone. In vivo studies with integrin-selected MDA-MB-231 subpopulations suggest that specific integrin expression may contribute to bone colonization versus soft organ metastasis (Tondravi et al., 1996), further supporting an interaction between BSP and HBC integrin expression.

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